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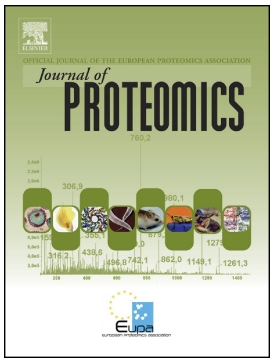

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Serum proteome changes and accelerated reduction of fat mass after Laparoscopic Gastric Plication in morbidly obese patients

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Bariatric surgery clinic and ethical approval

Patients underwent LGP at Laleh Hospital, Tehran, Iran. The institutional review board and the ethic committee of Shahid Beheshti University approved the study protocol for Human rights (IR.SBU.ICBS.97/1019).

Author Contributions

The manuscript was written with contributions from all authors. PS and AG conceptualized the overall study. PS wrote the draft version of the paper and revised it according to co-author comments. MT did the surgery. PS, AG and MT included patients. AS, PS, TBB and KK collected and analyzed the data. TBB, AS, and AG contributed to the data analysis and supervised the study. All authors have approved the final version of the manuscript.

Serum proteome changes and accelerated reduction of fat mass after Laparoscopic Gastric Plication in morbidly obese patients

Abstract

Laparoscopic Gastric Plication (LGP) is a relatively new bariatric surgical procedure which no part of the stomach is removed. It is not clearly understood how LGP leads to fatty tissue reduction. We aimed to investigate the impact of LGP on serum proteome and understand molecular mechanisms of LGP-induced weight loss post-surgery. A Prospective observational study of 16 obese individuals who underwent LGP was performed. A Label-free quantitative shotgun proteomics approach was used to compare serum proteome of subjects before surgery with serum of the same individuals 1 to 2 months post-surgery (T1) and 4 to 5 months post-surgery (T2). The proteome analysis revealed that 48 proteins were differentially regulated between pre-surgery and T1, and seven proteins between pre-surgery and T2 of which six proteins were shared between the two timepoints. Among differentially regulated proteins, four proteins (SRGN, FETUB, LCP1 and CFP) have not previously been described in the context of BMI/weight loss. Despite few differences following LGP, most regulated serum proteins are in accordance with alternative weight loss procedures. Pathway analysis revealed changes to lipid- and inflammatory pathways, including PPARα/RXRα, LXR/RXR and FXR/RXR activation, especially at T1. At T2, the pathways related to inflammation and immune system are most affected.

Significance

Among the available clinical therapies for morbid obesity, bariatric surgery is considered as the most effective approach to achieve long-term weight loss, alongside a significant improvement in metabolic syndrome. However, very little is known about the underlying mechanism associated with significant weight loss post-surgery. Understanding such mechanisms could lead to development of safer non-surgical weight loss approaches. We here present the first analysis of the impact of LGP on the serum proteome, to bring new insights into the underlying molecular mechanism. Our findings indicate that LGP has a comprehensive systemic effect based on the blood serum proteome profile which might account for accelerated reduction of fat mass after surgery, thus, food restriction is not the only reason for weight loss following this unique surgical approach. As secretory regions of the stomach are preserved in LGP and it is associated with minimal physiological and anatomical changes, the findings are of high importance in the field of bariatric surgery and weight loss.

Keywords Bariatric surgery; Laparoscopic Gastric Plication; Label free quantitation; Obesity; Proteomics

Introduction

The global prevalence of obesity has almost tripled since 1975 and has had an impact of $2.0 trillion for the worldwide economy [1-2]. Obesity is accompanied by systemic chronic low-grade inflammation, which may result in obesity-related comorbidities, such as type 2 diabetes and cardiovascular diseases [3]. Considering its growing prevalence and related medical problems, the necessity for successful treatments has been increased.

Bariatric surgery is the most effective treatment option for sustainable weight loss and improvement in comorbidities for morbidly obese individuals [4]. Roux-en-Y gastric bypass (RYGB) and *laparoscopic sleeve gastrectomy (LSG) are the most common techniques. However, both* suffer from complications in the form of staple line leakage or bleeding, which mainly arise from cutting the edge of the stomach [5].

By contrast, gastric plication (GP) with laparoscopic modification (LGP) reduces gastric volume by plication of the greater curvature of the stomach, that is without surgical removal of a part of the stomach which, to a large extent, mitigates complications. It was first described by Tretbar et al. 1976 in an animal study [6], and expanded with the laparoscopic modification by Talebpour and Amoli 2007 in a human study [7]. LGP is reversible, the gastric fundus is preserved, it is without foreign body placement and it is associated with minimal risk of nutrient deficiency, the common complication after RYGB [8-10]. Additionally, most of the weight loss following LGP is attributed to a loss of body fat mass with minimal fat-free mass reduction. Accordingly, it fulfills the ideal objective of any weight loss surgery in terms of body composition changes [11]. For all these reasons, LGP is an attractive alternative bariatric technique that is gaining increased usage [8]. In some reports, its complications and its effect on weight loss, on patient metabolic profile and on some selected gut hormones have been investigated [12-14]. However, as a relatively new technique, few studies have compared LGP with other well-established methods [8]. Therefore, it is still considered an investigational procedure.

It is now evident that food restriction is not the only reason for losing weight and improvements in obesity-related comorbidities after bariatric surgeries. It is of great importance to elucidate the underlying mechanism associated with significant weight loss post-surgery.

Previously, serum lipidomics in gastric bypass and banding were studied. It has been suggested that metabolic improvement after RYGB may be due to lipid profile alterations [15].

Given the lack of studies investigating the molecular systemic impact of LGP and bariatric surgery, we aimed to elucidate the systemic effects of this relatively new procedure on the molecular level, represented by the serum proteome. Blood serum bathes the internal organs and, as such, allows for studying the systemic impact of system perturbation [16]. Our hypothesis was that the effects of LGP, without gastric resection or bypass, extend beyond limitations in food intake due to restrictions of gastric volume. LGP, in contrast to LSG or RYGB, is associated with minimal physiological and anatomical changes [17-18], hence, studying the proteome profile changes could be informative.

To this end, we describe the systemic impact of LGP on the global serum proteome changes pre-surgery, using a label-free quantitative proteomics strategy, by comparing pre-surgery samples with 1 to 2 months post-surgery (T1) and 4 to 5 months post-surgery (T2). The present study provides deeper insight into systemic effects of LGP and its underlying molecular mechanism.

Materials and Methods

Study cohort and serum samples

A total of 16 obese subjects undergoing LGP by the same surgeon between November 2015 until May 2016 was consecutively recruited for this prospective observational study. The two-row LGP procedure was performed [17]. Subjects fulfilled the inclusion criteria if they were aged above 18 years with BMI>40 kg/m^2 or >35 kg/m^2 with at least one obesity-related comorbidity or >30 kg/m2 with metabolic syndrome. The exclusion criteria were as follows: any

psychological condition that could affect decision-making capabilities of the subjects (as defined by a psychiatrist), alcohol or drug abuse, uncontrolled malignant disease, and heart, renal or hepatic failure. All subjects were evaluated by a psychiatrist, an endocrinologist, a cardiologist, and a nutritionist before the surgical procedure. Before LGP, there was not any special diet for the subjects. After LGP, they took liquids (nutrient rich formulation) for 14 days, semi-liquids for 14 days, soft foods for 14 days and after that, normal foods with 800 kcal per day. Written consent was obtained and the study approved by the local ethical authorities.

Whole blood samples were collected after overnight fasting at three timepoints: pre-surgery, 1 to 2 months post-surgery (T1) and 4 to 5 months post-surgery (T2). It should be noted that seven subjects were excluded from analysis due to their unavailability at T2. Blood collection tubes were incubated at room temperature for 40 min, then centrifuged at 3000g for 10 min at 4°C. The serum was collected and stored in aliquots at -80°C.

Proteomics-sample preparation

Initially, all samples were randomized. Serum samples were depleted of the six most abundant proteins (albumin, IgG, IgA, antitrypsin, transferrin and haptoglobin) using the Agilent Multiple Affinity Removal column (4.6×50 mm) according to the manufacturer's instructions (Agilent Technologies, CA, USA). Filter-aided sample preparation protein digestion protocol was used to prepare samples for LC-MS [19-20]. The protein concentrations were measured with absorbance at 280nm (A280) using a NanoDrop 1000 UV−vis spectrophotometer (Thermo Scientific, Waltham, USA). 200µg of samples were transferred to YM-10 kDa spin filter (Millipore, Billerica, USA) and centrifuged at 14000g for 15 min at 4°C. A 400µl digestion buffer (0.5% sodium deoxycholate in 50mM triethylammonium bicarbonate) was added. Proteins were reduced with 10mM Tris (2-Carboxyethyl) phosphine and alkylated with 50mM

chloroacetamide for 20 min at 37°C. Samples were centrifuged at 14000g, 400µl of digestion buffer was added, followed by centrifugation. Protein digestion was carried out with 2µg sequencing-grade-modified trypsin (Promega, Madison, USA) diluted in 50µL digestion buffer and followed by overnight incubation at 37°C. The peptide material was recovered by centrifugation followed by phase separation, by adding 3:1 (v/v) ethyl acetate: sample volume and acidified with formic acid (FA) to a concentration of 1%. The tube was vortexed and centrifuged at 14000g for 1 min. The peptide-rich aqueous phase was collected, dried in a vacuum centrifuge, and stored at -80°C.

LC-MS/MS sample analysis

Prior to LC-MS/MS analysis, the peptides were resuspended in 2% acetonitrile, 0.1% FA and 0.1% trifluoroacetic acid. The samples were analyzed using a UPLC-nanoESI MS/MS setup with a NanoRSLC system (Dionex, CA, USA). The system was coupled online with an Objective PicoTip 360-20-10 emitter to a Q Exactive HF mass spectrometer (Thermo Scientific, Waltham, USA). All samples were analyzed in random order, in triplicates. Peptides were loaded onto a 2cm C18 trapping column (Dionex Acclaim PepMap RSLC C18) and separation was performed on a 75cm C18 reversed-phase column (Dionex Acclaim PepMap RSLC C18). Both were kept at 60°C. The peptides were eluted with a gradient of 98% solvent A (0.1% FA) and 2% solvent B (0.1% FA in acetonitrile) at a constant flow rate of 300nL/min. Solvent B was increased to 8% on a 5-min ramp gradient and to 30% on a 45-min ramp gradient. The mass spectrometer was operated in positive mode using a Top15 data-dependent MS/MS scan method. A full MS scan in the 375-1500 m/z range was acquired at a resolution of 120 000 with an AGC target of 3×10^6 and a maximum injection time of 50ms. Fragmentation of precursor ions was performed by higher-energy C-trap dissociation with a normalized collision energy of 27.

MS/MS scans were acquired at a resolution of 15000 with an AGC target of 2×10^5; maximum injection time was 100ms. Dynamic exclusion was set to 5s.

Data analysis

Mass spectrometry raw files were analyzed in MaxQuant version 1.6.0.1 and searched against the Uniprot human reference FASTA database (August 2017, containing 70,941 protein groups). Label-free protein quantitation (LFQ) algorithm was performed with a minimum ratio count of 1. Standard settings in MaxQuant were applied. A maximum of two tryptic missed cleavages was allowed. The false discovery rate (FDR) of identified proteins and peptides was set to a maximum of 1%, using a target-decoy fragment spectra search strategy. High confidence identifications were ensured. The "match between runs" feature was activated to transfer peptide identifications from one run to another, based on accurate retention time and mass.

Data processing

The output from MaxQuant was further filtered and processed in Perseus version 1.6.0.2. All reverse database hits and proteins identified only by site were removed. To ensure high quality quantitative data, additional filtering was performed for the 1% FDR filtered data: at least two unique peptides were required for a protein quantitation; the unique peptides were required to be quantifiable in at least 70% of samples. A principle component analysis was performed in MATLAB (R2018a) software to investigate replicate outliers. Missing values were imputed with values from normal distribution (width 0.3 and downshift 1.8) to simulate signals from low abundant proteins, using standard parameters in Perseus [21]. There were no replicate outliers.

Triplicate measurements of each sample were averaged in Perseus, and they were grouped according to timepoints: 16 samples pre-surgery, 16 at T1 and 9 at T2. Proteins with a

statistically significant abundance difference at any two timepoints were identified, using paired two sample t-test. To correct for multiple hypothesis testing, we applied permutation-based FDR control in Perseus using standard parameters (FDR:0.05, s0=0.1 and 250 randomizations).

Bioinformatics analysis

Differentially regulated proteins were functionally categorized based on gene ontology (GO) classification using WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) [22]. Identification of canonical pathways and relevant networks was performed with Ingenuity Pathway Analysis software (IPA; Ingenuity Systems, Redwood City, CA, www.ingenuity.com). Gene symbols and the corresponding protein fold change were imported to IPA software using core analysis. Standard settings for core analysis were employed, including: direct and indirect relationships between focused molecules, based on experimentally observed data (high confidence predictions and moderate confidence interactions excluded) were considered. Default settings of 35 molecules/network were employed. All sources of data from human, mouse and rat studies in the Ingenuity Knowledge Base were included. Because the serum proteome represents a sub-proteome, which in itself contains enriched categories, all identified proteins were used as background for GO and IPA. In addition to user dataset (all identified proteins), the ingenuity Knowledge Base (genes only) was utilized as a reference set to match proteins in our dataset with those in the Ingenuity Knowledge Base. Right-tailed Fisher's exact test was used to determine statistically significant pathways (p-value≤0.05). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [23] with the dataset identifier PXD010528

Validation of C-Reactive Protein and cytokine biomarkers quantitation

CRP and a number of cytokine biomarkers were further investigated with the Meso Scale Discovery (MSD) platform according to manufacturer's instructions. V-PLEX human CRP kit (MSD, Gaithersburg, USA) to measure CRP absolute concentration and proinflammatory panel 1 human kit (MSD, Gaithersburg, USA) to measure some cytokines, including IL-6, IL-8, IL-10, IFN-γ, and TNF-α were used. All standards and samples were measured in duplicate. MESO QuickPlex SQ 120 instrument was used to read the plates and data analysis was carried out using the MSD Discovery Workbench software.

Results

Study Cohort

A total of 16 obese subjects who underwent LGP, with the mean preoperative BMI of 41.2±5.3 kg/m^2 (33.1-48.5) and mean age of 37.9±10.2 years (22-48), was investigated to reveal the biological impact of weight loss due to LGP on human serum proteome. Subjects' characteristics are shown in Table 1. It should be noted that because of geographical relocation of seven patients and their unavailability at T2, they were excluded from the study at that time point. However, baseline characteristics of subjects at T1 and T2 were not significantly different. At T1, mean BMI decreased significantly from 41.2±5.3 to 36.6±4.9 kg/m^2 representing a 27% excess weight loss (EWL). The mean BMI measured at T2 was 32.5±5.4, with a 53.6% EWL.

Proteomics data overview

Protein digestion was performed on samples depleted of highly abundant proteins, using filter-aided sample preparation protocol. Randomized mass spectrometric data recorded in technical triplicate were subjected to protein identification and label free quantification. The workflow allowed us to identify 288 proteins. Following stringent filtering of the identified

proteins to ensure only proteins with high quality quantification were retained, 224 proteins met our quantitation criteria (Supplementary Table 1 in Ref [24]).

Quality control of proteomics data and cytokine biomarkers quantitation

As a quality control of proteomics data, CRP was chosen for further investigation. Absolute quantitative CRP levels in all of the pre- and post-surgery samples were measured using MSD assay. Box plot diagrams of CRP concentration in paired samples and correlation analysis considering all samples are shown in Fig. 1. Log2 fold change of CRP in MSD assay measurement was found to be -1.8 at T1 and -2.2 at T2 which has a similar pattern compared with LFQ measurement (-2.2 and -2.6 at T1 and T2, respectively). As indicated in Fig. 1B, a strong positive correlation between MSD assay and LFQ measurement was observed (R=0.975 and p-value=1.7e-26). The difference in CRP concentration between pre- and post-surgery samples, measured by both methods, is represented in Supplementary Fig. S1A.

A number of cytokines that are important in inflammation and immune response regulation were measured in five randomly selected samples. There was a significant reduction (p-value<0.05) in the level of IL-6 at T1 and T2 versus pre-surgery and significant increase (p-value<0.05) in the level of IL-8, IL-10, TNF-α, and INF-δ at T1 (Supplementary Fig. S1B-F).

Proteins with a Timepoint-specific abundance

Of the 224 quantifiable proteins, 48 (21.4%) had a statistically significant change in abundance (multiple testing correction by permutation-based FDR, FDR<0.05 and s0=0.1) at T1 compared with pre-surgery subjects: 23 proteins were less abundant and 25 were more abundant (Fig. 2A, Table 2). For T2, seven proteins were measured having an altered abundance: four upregulated and three downregulated (Fig. 2B, Table 2). Six proteins including SHBG, ITIH3,

NRP1, IL1RAP, PRG4 and CRP were identified as regulated at both timepoints of post-surgery and only one protein, FN1, was exclusively found at T2.

GO enrichment, Pathway and Network analysis

Gene ontology (GO) overrepresentation analysis for biological process and molecular function was conducted with WebGestalt to gain a biological view of significantly changing proteins [22]. The complete list of enriched GO terms is reported in Table 2 and Table 3 for T1 and T2 in Ref [24], respectively. The GO analysis revealed that most of the significantly changing proteins at T1 post-surgery have molecular functions such as lipid transporter activity, steroid binding and lipid binding. These proteins are enriched in biological processes related to protein and carbohydrate metabolism, whereas processes in the nervous system are mostly involved at T2.

To identify pathways and networks of interacting proteins, significant differentially abundant proteins with the corresponding fold changes were investigated using protein network information within IPA. To estimate the correlation of the network with the original list of focus proteins, a score based on IPA algorithms is determined, which considers the number of focus proteins and the size of the network [25]. Five significant networks were identified when pre-surgery subjects were compared with T1. The top enriched network defined as "Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry" with a score of 45, containing 18 differentially abundant proteins, is shown in Fig. 3A. Comparison of pre-surgery subjects with T2 showed a single protein-protein interaction network (Fig. 3B), containing seven focus molecules with a score of 21, which are related to "Cell-To-Cell Signaling and Interaction, Cell Death and Survival, Inflammatory Response". Direct and indirect molecular interactions are indicated by solid and dashed lines, respectively. Nodes in yellow correspond with upregulated

proteins and those represented in blue are downregulated. Nodes shown in gray are in our dataset but no significant differences in abundance were observed. Colorless nodes are suggested by IPA due to their relation with enriched proteins. Details of networks components are presented in Table 4 in Ref [24].

IPA was also used to connect significantly changing proteins to biological pathways. Firstly, we defined our identified proteins as a reference set. This revealed that alteration in the serum proteome at T1 correlates with alteration in eight enriched canonical pathways (p-value<0.05) (Fig. 3C). IPA analysis of differentially regulated proteins at T2 resulted in 11 enriched canonical pathways mainly involved in inflammation and the immune system (Fig. 3D). Left Y-axis shows –log of the P-value associated with each pathway, calculated by right-tailed Fischer's exact test. The orange line represents a threshold of 0.05. The right Y-axis depicts the ratio of the number of proteins in our dataset that are connected to the pathway over the total number of proteins in that pathway. Differentially regulated proteins associated with each pathway with their information are reported in Supplementary Table S1. Secondly, the Ingenuity Knowledge Base (Genes Only) was defined as a reference set to determine pathways; the obtained enriched pathways are shown in Supplementary Fig. S2A and S2B.

The Activation state of the pathways was predicted using Z-score. Positive Z-score values predict activation (orange bar), whereas a negative value indicates an inactivation or downregulation of an activity (blue bar). According to Z-score, predicted activity of PPARα/RXRα activation and LXR/RXR activation decreased at T1.

Discussion

The impact of LGP on weight loss and metabolic effects, such as improvement in diabetes and hypertriglyceridemia, have been investigated in the previous studies [17-18, 26].

Moreover, changes in some gastrointestinal hormones including ghrelin, GIP and GLP-1 have been previously evaluated [12, 27-28]. In the present research, untargeted proteomic approach was used to investigate unanticipated changes in serum protein profile following LGP. In a large cohort of subjects (800 cases), it has been shown that patients experience 60% EWL at 6 months after LGP [17], along with improved body composition due to accelerated reduction of fat mass [11]. Given that rapid weight loss will occur during the first months following surgery, we set up two timepoints during the first 6 months post-surgery.

Although LGP preserves the stomach fundus and does not include resection of secretory portions, we speculated that the reason for favorable effects of LGP, like other restrictive bariatric procedures, goes beyond the reduced volume of the stomach. Hence, it was expected that the effect of surgery would be reflected in the serum proteome, and the proteins would change by weight loss and the procedure.

We demonstrated for the first time that LGP, as a new and not well studied procedure, has a comprehensive systemic impact which, upon comparison with other studies, goes beyond impacts of calorie restriction. Quantitation of CRP by electrochemiluminescent immunoassay using MSD platform was performed as a quality control and indicative marker of the inflammation related proteomics data. CRP was found to be significantly changed following surgery, and is known that BMI has a significant contribution in the levels of circulating CRP, which is a marker of elevated adiposity [29]. Consistent with the mass spectrometry results, MSD assay showed a significant reduction in CRP levels for post-surgery subjects versus pre-surgery. As is clear from Fig. 1A, CRP, known as an inflammatory and risk marker for cardiovascular disease, is decreasing further at T2. We found a lower number of significantly changed proteins at T2 compared with T1. We ascribe this difference to be caused by i) many

serum-proteins having been returned to "normal" concentrations following surgical trauma and ii) seven pre-surgery subjects not being available at T2 and being excluded from analysis. Hence, the study has a reduced statistical power at T2.

The sparse nature of the surgical procedure limited the number of samples we could obtain for the study. Nonetheless, we evaluated whether T1 collection time, gender, and age had an impact on the findings. The results showed that when T1 is divided into 1 and 2 months, females and males, and age groups, a smaller number of significant proteins will be detected, but the overall significant proteins are roughly the same and did not change (Supplementary Table S2). However, based on the present study, we cannot exclude that there is a co-factor impact on the study. Oller Moreno et al. [30] has reported that males and females have different molecular responses to the diet-induced weight loss. In the present study, we were not able to detect such an impact, but given the uniqueness of the samples and therefore limited number of samples, we cannot exclude that the same is true for LGP.

Based on GO-analysis, most of the significantly changing proteins are involved in lipid- and carbohydrate metabolism. The two highest scoring networks, and hence the most affected networks by the procedure, identified using IPA at T1, network 1 and network 2, contained proteins involved in lipid metabolism and cardiovascular system development and function, respectively. It has been reported that RYGB has a significant role in improving cardiovascular functions which is mainly attributed to alterations in bile acid signaling, absorption and function [31]. It is worth noting that secretory regions of stomach are preserved in LGP, yet the procedure has a vast systemic impact with beneficial effects comparable with other surgeries. Among enriched pathways, PPARα/RXR α, LXR/RXR and FXR/RXR activation are most interesting and biologically relevant. LXRs, PPARs and FXRs are nuclear receptors with functions in lipid

and carbohydrate metabolism, as well as regulating bile acid and inflammatory response [32-34]. The specific mechanism by which these nuclear receptors contribute to glucose and lipid regulation by LGP should be investigated further.

IL-6 signaling was identified as one of the pathways at both timepoints. IL-6 was not identified in our proteomics study. Therefore, we investigated IL-6 levels in serum samples using the MSD-assay to ascertain whether it changed or not, and IL-6 was significantly reduced at both timepoints after surgery (p-value<0.05).

Differentially regulated proteins

Most of the differentially regulated proteins had been described in the context of weight loss due to diet and/or bariatric surgery and BMI (Table 3). For instance, Neurophilin-1 (NRP1) was found to be significantly upregulated at both timepoints postoperatively. Similarly, Geyer et al. reported a significant increase in NRP1 levels after diet-induced weight loss [35]. Our report is the first to link NRP1 with weight loss following bariatric surgery. Interestingly, it has been reported very recently that NRP1, as expressed by macrophages, has a vital role in limiting obesity-associated inflammation and regulating fatty acid uptake in macrophages [36]. Reduction in the level of inflammatory markers such as CRP [35, 37] and SAA1[35, 38], and anti-inflammatory markers increment such as ADIPOQ [39-40], IL1RAP [35, 41] and CD14 [35], which all are in accordance with previous findings are shown in Table 3. The link between obesity and metabolic syndrome has been attributed to the inflammatory process [42], and bariatric surgery is known to be effective in reduction of obesity-associated inflammation [37]. In addition to ADIPOQ, significant changes in the level of three other adipokines including RBP4, SRGN and SERPINF1 have been detected in our untargeted MS-based shotgun proteomics shown in Table 3. Moreover, significant reduction in pro-inflammatory IL-6 cytokine

at T1 and T2 and significant increase in anti-inflammatory IL-10 cytokine at T1 have been determined by MSD immunoassay. Modification in the level of these inflammatory and anti-inflammatory markers may reflect improvement in the low-grade inflammation associated with obesity and can be attributed to the improvement in metabolic syndrome following LGP.

In addition to the proteins known to be affected by weight loss, among the 48 proteins showing differential regulation at T1, 11 serum proteins had not previously been put into context with BMI, bariatric surgery or weight loss. As mentioned, we expected the serum proteins to be altered due to i) the weight loss/change in BMI, and ii) the surgery trauma which is especially present at T1. Of the 11 proteins, four proteins (SRGN, FETUB, LCP1, and CFP) appear to be changed by weight loss and BMI, while the rest showed alteration due to surgery, based on protein functions and previous reports.

SRGN showed a significant increase postoperatively and is a new adipocytokine, highly expressed in adipocytes of epicardial adipose tissue. It is not yet clear what cell type and tissue account for circulating SRGN. It is proposed to be systemically involved in adipose tissue inflammation [43]. FETUB has been described as an adipokine/hepatokine which is increased in patients with insulin resistance/diabetes [44]. In a recent study comparing plasma protein profile of two obese cohorts from Canada and European countries, FETUB was identified as a gender-associated protein [45]. It showed a significant reduction following LGP. LCP, with a role in the regulation of leukocyte adhesion, is hypothesized to correlate with body mass gain based on a study of mice on a high fat diet [46]. We found a significant increase in its circulating level postoperatively. CFP has been described as being involved in the alternative complement activation. CFP also contributes to altered lipid metabolism, insulin resistance and low grade inflammation [47]. We observed its reduction postoperatively.

The amount and speed of weight reduction following LGP, is much higher than the diet-induced weight loss. Moreover, patients experience improvement in diabetes and glucose homeostasis following LGP, before occurring a significant weight reduction. Hence, the reason for accelerated weight loss and improvement in metabolic parameters are not only due to the gastric volume reduction; in fact, underling mechanism in LGP could be different from the diet-induced weight loss. According to the findings, most of the regulated proteins are shared between LGP and dietary intervention. This shows that LGP does not result in unusual impacts on the global serum proteome and it can be safe regarding the dynamic changes in the serum proteome. On the other hand, these regulated proteins, except nine proteins in overall 49 significant proteins, also have found following weight loss by other types of bariatric surgeries (Table 3).

Recent study by Albrechtsen et al. [48], investigating dynamics of plasma proteome after RYGB revealed changes in inflammatory and lipid homeostasis markers. This study compared with the research on the caloric restriction (Cambridge diet) carried out by the same group [35] showed a significant overlap of inflammatory proteins. By contrast, a differently regulated lipid homeostasis system was identified between two interventions.

In our research, some of the proteins in the inflammatory panel identified by the above-mentioned group including CRP, CD14, CFI, C7, CFI, C4BPA, FN1, SAA1, and SERPINF2 were found. Based on the results carried out by that group, LDL-associated proteins have been regulated in a similar direction in both interventions, while HDL-associated proteins such as APOA1, APOA2, APOA4 and APOM were only increased after RYGB. Contrary to the mentioned group's results, we found reduction in the level of these apolipoproteins. We attributed this inconsistency to the time of study, as they found the increase after 52 and 104

weeks following surgery. In another study which inspected the difference between gastric bypass and low-calorie diet [49], pathway analysis revealed that regulated proteins influence inflammation and lipid metabolism in both interventions, while carbohydrate and protein metabolism have been affected in surgery intervention. These results are in line with our findings from pathway and network analysis after LGP.

Exclusively identified protein at timepoint T2

FN1 was the only protein with a statistically significant reduction identified exclusively at T2. It has been shown that FN1 is one of the dysregulated adipocyte-specific genes in obese adipose tissue and its plasma level has a positive correlation with BMI and triglyceride concentration in overweight subjects [50-51]. For the first time, we have found its connection to surgery-induced weight loss with an expected reduction in its levels.

Proteins with opposite regulation pattern compared with known protein changes

We found eight proteins whose directionality of abundance differences were in contrast with previous reports. The levels of THBS1, SPARC, VWF, and C4BPA were significantly increased at T1. THBS1 plays a role in proinflammatory adipokines production. Its serum level is correlated with the degree of obesity and has been found to be decreased one year after laparoscopic sleeve gastrectomy [52]. SPARC, as the first extracellular matrix protein described in adipose tissue, was reported to have been significantly reduced after 9 months of RYGB [53]. The observed difference of these two proteins might be due to the time of the study. Long-term follow up is required to indicate the reason for this inconsistency. VWF, as a marker of endothelial dysfunction, was reported to be significantly downregulated after RYGB [54]. The effect of bariatric surgery on the markers of endothelial function was described in the literature

[55]. It has been proposed recently that the weight loss is not the only reason for endothelial function improvement; other effects of RYGB may be involved [54]. More studies are required to further explore the VWF changes following bariatric surgeries. C4BPA, which is known as an inhibitor of classical and lectin complement pathways, can bind to CRP and decrease the complement activation [56]. In a recent study investigating the plasma proteome changes after RYGB, the levels of C4BPA have been significantly reduced [48]. In contrast to our study, the levels of CRTAC1, APOA1, APOA2, and APOA4 have been significantly increased in the recent mentioned study [48]. We attributed this inconsistency to the time of study, as they found the increase at the first weeks following surgery (2 weeks) or after 52 and 104 weeks.

Of note, RYGB and LSG are associated with dramatic anatomical and physiological changes. For that reason, it is possible that the above-mentioned opposite changes are as a result of the type of surgery.

Conclusions

We present the first study that provides a broader insight into the dynamic changes in the serum proteome indicative of systemic low-grade inflammation reduction due to LGP-induced weight loss. The vast systemic impact of LGP, based on the blood serum proteome profile, supports the hypothesis that food restriction is not the only reason for weight loss following LGP, and it might account for accelerated reduction of fat mass.

It is an interesting finding since LGP preserves the secretory regions of the stomach and is associated with minimal physiologic changes in the gastrointestinal tract. A panel of significantly altered proteins were reported in previous studies and four novel affected proteins in the context of BMI/weight loss, including SRGN, FETUB, LCP1 and CFP, have been found. Based on pathway and Go analysis results, LGP has the potential to change proteins involved in

lipid metabolism, inflammation and carbohydrate metabolism. Of importance, our findings contribute to existing weight loss proteomic studies and, in addition, may explain that reported metabolic improvement after LGP may occur from a similar mechanism to other weight loss strategies. However, bearing in mind the small sample size as a limitation of our research that may make the study as a hypothesis-generating pilot study, we suggest further research with larger populations and evaluate any possible co-factor effects such as gender dependency and also comparing different surgical approaches. This will lead to in-depth understanding of possible differences in surgically induced weight loss effects on human blood proteome and associated mechanism.

Associated Content

- **Supporting Information**

Supplementary Table S1. Differentially regulated proteins associated with the significantly enriched canonical pathways. (Word)

Supplementary Table S2. Data related to T1 divided into 1 and 2 months, females and males, and age groups. (Excel)

Supplementary Fig S1. (A) Difference in CRP concentration between pre- and post-surgery samples determined by proteomics LFQ intensity and MSD assay. (B-F) Concentration of indicated cytokines determined using MSD assay. p-values are presented using paired-t test. (Word)

Supplementary Fig S2. Canonical pathways affected by differentially regulated proteins using Ingenuity Knowledge Base (Genes Only) as a reference set, at A) T1 B) T2. (Word)

- **Accession Codes**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [23] with the dataset identifier PXD010528.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Figure legend

Fig. 1. (A) Box plot of CRP concentration in paired samples according to proteomics LFQ intensity and MSD assay. P-values calculated by paired two-sample t-tests. (B) Correlation analysis between proteomics and MSD assay measurement. Pearson correlation and p-value are shown.

Fig. 2. (A) Volcano plots of protein changes comparing T1 and pre-surgery, and (B) T2 and pre-surgery. Each dot depicts a quantified protein, and the statistically significant proteins have been highlighted in red. The black curve represents the threshold of significance of FDR < 0.05 after applying permutation-based FDR control at s0=0.1.

Fig.3. (A) The top-scoring network when pre-surgery subjects were compared with T1, and (B) The only identified network when pre-surgery subjects were compared to T2. Direct and indirect molecular interactions are indicated by solid and dashed lines, respectively. Nodes in yellow correspond with upregulated proteins and those represented in blue are downregulated. Nodes shown in gray are in our dataset but no significant differences in abundance were observed. Colorless nodes are suggested by IPA due to their relation with enriched proteins C) Canonical pathways affected by differentially regulated proteins at T1, and (D) T2. Left Y-axis shows –log of the P-value associated with each pathway, calculated by right-tailed Fischer's exact test. The orange line represents a threshold of 0.05. The right Y-axis depicts the ratio of the number of proteins in our dataset that are connected to the pathway over the total number of proteins in that pathway.

Table 1. Clinical and demographic characteristics of study subject

Variable	Pre-surgery (n=16)	T1 (n=16)	T2 (n=9)	p-value pre vs T1	p-value pre vs T2
Age (years)	37.9 ± 10.2	-	36.9 ± 11.9	-	-
Gender ratio, (F:M)	11:5	11:5	6:3	-	-
BMI (Kg/m2)	41.2 ± 5.3	36.6 ± 4.9	32.5 ± 5.4	<0.0001	<0.0001
%EWL	-	27 ± 6.3%	53.6 ± 15%	-	-
Triglycerides (mg/dl)	130.63 ± 53.78	106.88 ± 40.98	91.89 ± 24.7	<0.05	<0.05
Cholesterol (mg/dl)	176.69 ± 36.20	166.38 ± 28.77	165.89 ± 30.25	NS	NS
HDL-C (mg/dl)	45.5 ± 12.44	39.31 ± 8.38	46.67 ± 7.31	<0.05	NS
LDL-C (mg/dl)	105.23 ± 27.03	105.25 ± 23.93	101 ± 27.34	NS	NS
Base-line comorbidity		-	-	-	-
Hypertension	3 (18.7%)				
Dyslipidemia	8 (50%)				
Depression	3 (18.7%)				

Values are mean ± standard deviation, or the number of subjects
p-values are presented using paired-t test
BMI body mass index, LDL-C low-density lipoprotein cholesterol, HDL-C high-density lipoprotein Cholesterol, NS Not Significant, EWL% percent excess weight loss
EWL%= (preoperative weight – weight at each visit/excess weight × 100%)

Table 2. Significantly changed serum proteins (q-value <0.05) post- vs pre-surgery

Protein IDs	Protein names	Gene names	-log $_{10}$ p-value	Fold change log2 (post vs pre)
T1 vs pre-surgery				
P04278	Sex hormone-binding globulin	SHBG	8.75	1.16
P10124	Serglycin	SRGN	2.58	1.10
P20742	Pregnancy zone protein	PZP	1.71	1.06
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	8.76	0.87
Q15848	Adiponectin	ADIPOQ	1.78	0.81
Q13790	Apolipoprotein F	APOF	7.89	0.80
O14786	Neuropilin-1	NRP1	6.65	0.74
P05090	Apolipoprotein D	APOD	2.35	0.67
P04003	C4b-binding protein alpha chain	C4BPA	1.75	0.61
P13796	Plastin-2	LCP1	5.50	0.59
Q9NPH3	Interleukin-1 receptor accessory protein	IL1RAP	4.45	0.57
P16930	Fumarylacetoacetase	FAH	2.03	0.54
P09172	Dopamine beta-hydroxylase	DBH	1.61	0.54
Q13201	Multimerin-1	MMRN1	3.64	0.47
Q14126	Desmoglein-2	DSG2	4.01	0.46
O95445	Apolipoprotein M	APOM	6.03	0.44
P07359	Platelet glycoprotein Ib alpha chain	GP1BA	3.62	0.38
P04275	von Willebrand factor;von Willebrand antigen 2	VWF	2.01	0.36
P08697	Alpha-2-antiplasmin	SERPINF2	2.06	0.36
P12955	Xaa-Pro dipeptidase	PEPD	2.30	0.33
P19823	Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2	4.27	0.31
P08571	Monocyte differentiation antigen CD14	CD14	4.67	0.31
P07996	Thrombospondin-1	THBS1	2.77	0.28
P09486	SPARC	SPARC	3.73	0.27
P10643	Complement component C7	C7	5.27	0.216
Q99969	Retinoic acid receptor responder protein 2	RARRES2	3.93	-0.30
P02647	Apolipoprotein A-I	APOA1	2.82	-0.31
P05156	Complement factor I	CFI	5.994	-0.32
P00742	Coagulation factor X	F10	3.77	-0.32
P02766	Transthyretin	TTR	5.48	-0.36
P02652	Apolipoprotein A-II	APOA2	2.87	-0.38
P02753	Retinol-binding protein 4	RBP4	3.37	-0.38
P08709	Coagulagction factor VII	F7	3.32	-0.39
P36955	Pigment epithelium-derived factor	SERPINF1	4.94	-0.39
P80108	Phosphatidylinositol-glycan-specific phospholipase D	GPLD1	5.12	-0.46
Q6UXB8	Peptidase inhibitor 16	PI16	1.81	-0.52
P27918	Properdin	CFP	6.12	-0.53

P14780	Matrix metalloproteinase-9	MMP9	1.46	-0.62
P02774	Vitamin D-binding protein	GC	1.66	-0.63
Q92954	Proteoglycan 4	PRG4	7.12	-0.707
P06727	Apolipoprotein A-IV	APOA4	2.52	-0.71
Q96KN2	Beta-Ala-His dipeptidase	CNDP1	6.53	-0.78
Q9UGM5	Fetuin-B	FETUB	5.67	-0.789
P02743	Serum amyloid P-component	APCS	3.04	-0.85
Q9NQ79	Cartilage acidic protein 1	CRTAC1	1.76	-1.06
P02741	C-reactive protein	CRP	1.76	-1.08
P0DJI8	Serum amyloid A-1 protein	SAA1	2.46	-1.391
P68871	Hemoglobin subunit beta	HBB	5.22	-1.45
	T2 vs pre-surgery			
Q92954	Proteoglycan 4	PRG4	3.69	-0.83
O14786	Neuropilin-1	NRP1	2.89	0.68
P04278	Sex hormone-binding globulin	SHBG	3.80	0.96
P02741	C-reactive protein	CRP	2.85	-2.6
P02751	Fibronectin	FN1	3.06	-0.70
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	4.95	0.82
Q9NPH3	Interleukin-1 receptor accessory protein	IL1RAP	2.92	0.59

Table 3. Significantly altered abundance proteins compared to literature

Gen names	Direction of change in our study	Previously reported	
		Direction of change/ Context	Reference
SHBG	↑	↑/ Diet- and surgery-induced weight loss (RYGB and SG)	[30, 35, 48, 57]
SRGN	↑	-	-
PZP	↑	↑/ Diet-induced weight loss	[58]
ITIH3	↑	↑/ Diet- and surgery-induced weight loss (RYGB)	[30, 35, 48]
ADIPOQ	↑	↑/ Diet- and surgery-induced weight loss (RYGB)	[30, 39-40, 48]
APOF	↑	↑/ Diet-induced weight loss	[35]
NRP1	↑	↑/ Diet-induced weight loss	[35]
APOD	↑	↑/ surgery-induced weight loss (RYGB)	[48, 59]
C4BPA	↑	↓/ surgery-induced weight loss (RYGB)	[48]
LCP1	↑	-	-
IL1RAP	↑	↑/ Diet- and surgery-induced weight loss (RYGB)	[35, 41]
FAH	↑	-	-
DBH	↑	↑/ Diet-induced weight loss	[30]
MMRN1	↑	-	-
DSG2	↑	-	-
APOM	↑	↑/ Surgery-induced weight loss (RYGB)	[48]
GP1BA	↑	↑/ Diet-induced weight loss	[35]
VWF	↑	↓/ Diet- and surgery-induced weight loss (RYGB)	[54, 60]
SERPINF2	↑	↑/ Diet-induced weight loss	[35]
PEPD	↑	-	-
ITIH2	↑	↑/ Diet-induced weight loss	[30, 35]
CD14	↑	↑/ Diet-induced weight loss	[35]
THBS1	↑	↓/ Surgery-induced weight loss (SG)	[52]
SPARC	↑	↓/ Surgery-induced weight loss (RYGB)	[53]
C7	↑	↑/ Diet- and surgery-induced weight loss (RYGB)	[30, 35, 48]
RARRES2	↓	↓/ Diet- and surgery-induced weight loss (RYGB, SG)	[61-62]
APOA1	↓	↓/ Diet-induced weight loss ↑/Surgery-induced weight loss (RYGB)	[35, 48]
CFI	↓	↓/ Diet- and surgery-induced weight loss (RYGB)	[30, 35, 48]
F10	↓	↓/ Diet- and surgery-induced weight loss (RYGB)	[48, 63-64]
TTR	↓	↓/ Diet- and surgery-induced weight loss (RYGB, SG)	[35, 48, 65]
APOA2	↓	↓/ Diet- induced weight loss ↑/Surgery-induced weight loss (RYGB)	[35, 48]
F7	↓	-	-
RBP4	↓	↓/ Diet- and surgery induced weight loss (gastric banding, RYGB)	[35, 48, 66]
SERPINF1	↓	↓/ Diet- and surgery-induced weight loss	[35, 48, 67]

		(SG, RYGB)	
GPLD1	↓	↓/ Diet- and surgery-induced weight loss (RYGB)	[35, 48]
PI16	↓	-	-
CFP	↓	-	-
MMP9	↓	↓/ Surgery-induced weight loss (gastric banding)	[68]
GC	↓	↓/ Surgery-induced weight loss (SG)	[67]
PRG4	↓	↓/ Diet- and surgery induced weight loss (RYGB, SG)	[35, 48, 69]
APOA4	↓	↓/Diet- and surgery-induced weight loss (SG, RYGB) ↑/surgery-induced weight loss (RYGB)	[35, 48, 70-71]
CNDP1	↓	↓/ Diet- and surgery-induced weight loss (RYGB)	[35, 48]
FETUB	↓	-	-
APCS	↓	↓/ Diet- and surgery-induced weight loss (RYGB)	[35, 48]
CRTAC1	↓	↑/ Surgery-induced weight loss (RYGB)	[48]
CRP	↓	↓/ Diet- and surgery-induced (SG, RYGB and gastric banding)	[35, 37, 48]
SAA1	↓	↓/ Diet- and surgery-induced weight loss (RYGB)	[35, 38, 48]
HBB	↓	-	-
FN1	↓	↓/ Diet-induced weight loss	[35]

Significance

Among the available clinical therapies for morbid obesity, bariatric surgery is considered as the most effective approach to achieve long-term weight loss, alongside a significant improvement in metabolic syndrome. However, very little is known about the underlying mechanism associated with significant weight loss post-surgery. Understanding such mechanisms could lead to development of safer non-surgical weight loss approaches. We here present the first analysis of the impact of LGP on the serum proteome, to bring new insights into the underlying molecular mechanism. Our findings indicate that LGP has a comprehensive systemic effect based on the blood serum proteome profile which might account for accelerated reduction of fat mass after surgery, thus, food restriction is not the only reason for weight loss following this unique surgical approach. As secretory regions of the stomach are preserved in LGP and it is associated with minimal physiological and anatomical changes, the findings are of high importance in the field of bariatric surgery and weight loss.

Highlights

- The systemic impact of LGP on the global serum proteome was investigated.
- A panel of significantly altered proteins including some novel proteins was detected.
- LGP has the potential to change proteins involved in lipid metabolism and inflammation.
- Food restriction is not the only reason for weight loss following LGP.